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Phosphorylation and Activation of Protamine Kinase by Two Forms of a Myelin Basic Protein Kinase from Extracts of Bovine Kidney Cortex*

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Two myelin basic protein kinases designated MBPK-1 and MBPK-2 were purified to apparent homogeneity from extracts of bovine kidney cortex. The purified preparations exhibited an apparent $M_t \approx 40,000$, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 42,000 (MBPK-1) and 45,000 (MBPK-2) by gel permeation chromatography. Up to 0.4 and 1.8 mol of phosphoryl groups were incorporated per mol of MBPK-1 and MBPK-2, respectively, on threonines following incubation with ATP. Autophosphorylation, incubation with protein phosphatase 2A (PP2A), CD45, or T-cell protein tyrosine phosphatase did not affect MBPK-1 activity. Autophosphorylation increased by about 3-fold MBPK-2 activity. This autophosphorylation and activation was reversed by PP2A but not by CD45 or T-cell protein tyrosine phosphatase. MBPK-1 and MBPK-2 displayed a positive reaction with an antibody to mitogen-activated protein kinase. Purified preparations of protamine kinase were activated by about 1.5–6-fold and, after inactivation with PP2A, were reactivated by about 30% by MBPK-1 and MBPK-2. Activation and reactivation correlated with the incorporation, respectively, of 0.1–0.5 and 0.5 mol of phosphoryl groups/mol of the protamine kinase on serines. The results show that MBPK-1 and MBPK-2 are protamine kinase-activating kinases and suggest that MBPK-1 and MBPK-2 may be related to mitogen-activated protein kinase.

Mitogen-activated protein kinase (MAP kinase)* represents a family of protein serine/threonine kinases (1–6) that exhibit an apparent $M_t \approx 40,000$–48,000 (7–12). These kinases are activated as a rapid intracellular response to a variety of extracellular signals including insulin, epidermal growth factor, platelet-derived growth factor, fetal growth factor, nerve growth factor, interleukin-1, and phorbol esters (1–12). This activation is unusual because it proceeds via phosphorylation of MAP kinase on tyrosine (e.g. 13–15) and either (e.g. 13–15) or both (10) threonine and serine residues. In mitogen-treated cells, phosphorylation of MAP kinase on tyrosines and threonines appears to be catalyzed by an enzyme termed MAP kinase kinase (16–18) which itself appears to be activated by the serine threonine Raf-1 kinase (19, 20). One function of MAP kinase may be to trigger the phosphorylation and activation of other protein kinases. This possibility is indicated by the observations that MAP kinase phosphorylated and activated purified preparations of a distinct insulin- and mitogen-activated ribosomal protein S6 kinase of apparent $M_t \approx 90,000$ (21) and a new glycogen synthase kinase composed of two polypeptides of apparent $M_t \approx 60,000$ and 53,000 (22).

The major cytosolic protein kinase in bovine kidney is a distinct enzyme of apparent $M_t \approx 45,000$ (23). In vitro, this enzyme acts on protein synthesis initiation factor 4E (24) and ribosomal protein S6 (25). Insulin stimulated the activity of the protamine kinase in isolated rat hepatocytes (26). Thus, this enzyme could contribute to the insulin-stimulated phosphorylation of initiation factor 4E (27, 28) and ribosomal protein S6 (29–31). The protamine kinase itself appeared to be regulated by phosphorylation. Thus, following incubation with PP2A, purified preparations of the protamine kinase were inactivated (32, 33). By contrast, purified preparations of other protein phosphatases including the major cytoplasmic protein serine/threonine phosphatases protein phosphatase 1, protein phosphatase 2B, and protein phosphatase 2C were without effect on protamine kinase activity (33). These observations indicated that PP2A was a specific protamine kinase-inactivating phosphatase (33). However, information on protamine kinase-activating kinases has been lacking.

Based on similarities in the kinetics of insulin stimulation, the protamine kinase and MAP kinase may also be linked in a cascade (26, 32). To examine this possibility, we set out to purify MAP kinase from bovine kidney, the tissue source used to purify the protamine kinase. We employed MBP as substrate (34) and as described earlier (7, 8, 10, 35), chromatography on phenyl-Sepharose was employed to remove other MBP kinases. Two peaks of MAP kinase designated MBPK-1 and MBPK-2 were resolved by chromatography on protamine-agarose. In this paper, we describe the purification of MBPK-1 and MBPK-2 to apparent homogeneity and show that these enzymes phosphorylate and activate the protamine kinase. However, although MBPK-1 and MBPK-2 reacted with an antibody that recognizes the MAP kinases ERK-1 and ERK-2 (11, 12), other properties of MBPK-1 and MBPK-2 distinguish these enzymes from MAP kinases.

**EXPERIMENTAL PROCEDURES**

Materials—Mouse anti-MAP kinase monoclonal antibody generated to a peptide derived from a 21 amino acid sequence (residues
325–345) of ERK-1 and ERK-2 (11, 12) was from Zymed corp. Mouse anti-phosphotyrosine monoclonal antibody, the synthetic peptide inhibitor of protein kinase C (RARKGALRQKVN), the src family protein tyrosine kinase pS64, and the rabbit anti-S6 kinase polyclonal antibody which recognizes the 70- (36) and 90-kDa (37) S6 kinase varieties, were form Upstate Biotechnology Inc. A preparation of phosphotyrosine monoclonal antibody was obtained from ICN Biochemicals. Goat anti-mouse and anti-rabbit IgG were from Bio-Rad. Phenyl-Sepharose, protein A-Sepharose, and the synthetic peptide inhibitor of protein kinase A (TYYADFIAS-GRTGRNIAHID) were from Sigma. Histone H1, histone H2A, histone H2B, histone H3, and histone H4 were from Boehringer Mannheim. Proteamine kinase (23), PP2A2 (33), and MBP (38) were purified to apparent homogeneity as described. The PT-PPase CD54 (39) and T-cell PT-PPase (40) were a gift from Dr. Edmond H. Fischer (University of Washington, Seattle). A preparation of T-cell PT-PPase was also obtained from Dr. Nicholas Tonks (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). Other materials are given in Refs. 23, 24, and 32.

Determination of MBP Kinase Activity—The assay mixture (0.05 ml) contained 50 mM Tris-chloride, pH 7.0, 10% glycerol, 0.175 mg of bovine serum albumin, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM MgCl2, 0.2 mM [γ-32P]ATP (1000 cpm/pmol), 10 mM MgCl2, and MBP sample. The ATP and MgCl2 were added after equilibration of the mixture for 5 min at 30 °C in a plastic microcentrifuge tube. After a 10-min reaction period, 1 ml of 12% trichloroacetic acid was added, and the mixture was centrifuged (15,000 g for 1 h at 4 °C). The supernatant fluid was discarded, and the pellet was washed five times with 1 ml portions of trichloroacetic acid. Aqueous counting scintillant was added to the tube, and the radioactivity was determined.

Kinase sample was quantitated from control incubations. One unit of MBP kinase activity was defined as the amount of enzyme that incorporated 1 nmol of phosphoryl groups into MBP/min. To ensure linearity, the extent of phosphoryl group incorporation was limited to <1 nmol.

Anti-phosphotyrosine monoclonal antibody, the synthetic peptide in-...
were combined separately (step 6), and each pool was then applied onto a separate column (2 x 10 cm) of phenyl-Sepharose equilibrated in buffer B containing 0.25 M NaCl. Each column was washed with 300 ml of this buffer and then developed with a 300-ml linear gradient from buffer B containing 0.25 M NaCl to buffer C. The activities of MBPK-1 and MBPK-2 were eluted at about 25 and 50% ethylene glycol, respectively. Active fractions containing MBPK-1 and MBPK-2 were pooled separately (step 7), diluted with 5 volumes of buffer B and then each solution was applied onto a separate column (1.8 x 3 cm) of CM-Sepharose equilibrated with buffer B. The columns were washed with 200 ml of buffer B, and the eluent from each column was applied onto a separate column of protamine-agarose (1.8 x 3 cm) equilibrated in buffer B. Each protamine-agarose column was washed with 50 ml of buffer B, and MBPK-1 and MBPK-2 were recovered with buffer B containing 1.0 M NaCl. Active fractions containing MBPK-1 and MBPK-2 were combined separately (≈2 ml), and each pool was applied onto a separate calibrated column (2.5 x 95 cm) of Sephacryl S-200 equilibrated and developed in buffer B containing 0.2 M NaCl and 0.1% Triton X-100. The active fractions from each column (each about 18 ml) were pooled (step 8) and concentrated separately on a small column (1 x 2 cm) of protamine-agarose as described above. Active fractions containing MBPK-1 and MBPK-2 were pooled separately, and then each pool was dialyzed overnight with three changes against 20 volumes of buffer B and then for 6 h against 20 volumes of buffer B containing 50% glycerol. The solutions were then aliquoted and stored at -20°C. A summary of the purification is presented in Table I.

TABLE I

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Specific Activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract</td>
<td>4,500</td>
<td>119,000</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>2. DEAE-cellulose</td>
<td>1,200</td>
<td>26,400</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>3. Q-Sepharose</td>
<td>600</td>
<td>13,800</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>4. Phenyl-Sepharose</td>
<td>800</td>
<td>1,980</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>5. Protamine-agarose</td>
<td>800</td>
<td>1980</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>6. Poly(lysine)-agarose</td>
<td>145</td>
<td>38</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>7. Phenyl-Sepharose</td>
<td>105</td>
<td>4.4</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>8. Sephacryl S-200</td>
<td>112</td>
<td>1.7</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

**Homogeneity and Composition**—Purified preparations of MBPK-1 and MBPK-2 exhibited an apparent Mₐ = 40,000, as determined by SDS-PAGE (Fig. 2). MBPK-1 and MBPK-2 exhibited an apparent Mₐ = 42,000 and 45,000, respectively, as determined by gel permeation chromatography on Sephacryl S-200 (Fig. 2). The purification procedure has been employed successfully 12 times. In three of the purified preparations of MBPK-1 and MBPK-2, a minor protein contaminant (about 5%) of apparent Mₐ = 60,000 was detected by silver staining (44). This protein was not phosphorylated when autophosphorylation was examined.

**RESULTS**

**Autophosphorylation**—Up to 0.4 and 1.8 mol of phosphoryl groups was incorporated per mol of MBPK-1 and MBPK-2, respectively, following incubation of the purified preparations with Mg²⁺ (1 mM) and [γ-32P]ATP (0.2 mM) (Fig. 3). The optimal concentration for Mg²⁺ for this reaction was 1 mM. At 10 mM Mg²⁺, autophosphorylation was inhibited by about 40%. In the presence of 1 or 10 mM Mg²⁺, 2 mM Mn²⁺ inhibited the rate of autophosphorylation also by about 40%. Sodium vanadate (0.2 mM), ammonium molybdate (40 μM), or microcystin-LR (0.4 μM) had little or no effect on the rate and extent of autophosphorylation of MBPK-1 or MBPK-2 (not shown). Phosphoamino acid analysis of six different preparations showed that autophosphorylation of MBPK-1 and MBPK-2 occurred on threonine residues (e.g. Fig. 4). Similar results were obtained when the protein tyrosine phosphatase inhibitors sodium vanadate (0.2 mM) or ammonium molybdate (40 μM) were included in the autophosphorylation reactions.

Autophosphorylation had little effect, if any, on the activity of MBPK-1 with MBP (Fig. 3), histone H1, histone H2A, and histone H2B as substrates. Incubation with PP2A, CD45, or T-cell PTPase had little effect, if any, on MBPK-1 activity before or after autophosphorylation (Fig. 5A). Autophosphorylation of MBPK-1 was reversed, however, by incubation with PP2A, but not with CD45 or with T-cell PTPase (Fig. 5C).

Autophosphorylation was accompanied by a ~3-fold increase in the activity of MBPK-2 with MBP (Fig. 3), histone
Fractions of 1.8 ml were collected. The active fractions (-2-3 ml) of MBPK-1 on Sephacryl S-200 were determined with ferritin and bovine serum albumin, ovalbumin, and ribonuclease. The eluate from phenyl-Sepharose was then applied onto a calibrated column (2.5 cm) of Sephacryl S-200 equilibrated and developed in buffer B containing 0.2 mM NaCl and 0.1% Triton X-100. The activity (●) was determined as described under "Experimental Procedures." The absorbance at 595 nm (○) was determined according to Bradford (43) using 50-µl aliquots. The flow rate was 20 ml/h. The bars indicate the fractions that were pooled. The protein standards is indicated times, a 5-µl aliquot of the incubations was assayed for MBP kinase activity as described under "Experimental Procedures." The absorbance at 595 nm (○) was determined according to Bradford (43) using 50-µl aliquots. The flow rate was 20 ml/h. The bars indicate the fractions that were pooled. The protein standards were bovine serum albumin, ovalbumin, and ribonuclease. V1 was determined with ferritin and V2 with FMN. Panel C shows the SDS-PAGE pattern of 5 µg of MBPK-1 (lane 1) and MBPK-2 (lane 2) from Sephacryl S-200 after concentration on protamine-agarose as described in the text. The gel was stained with Coomassie Blue. The position of marker proteins M, × 10^3 from top to bottom is phosphorlyase, bovine serum albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor.

H1, histone H2A, and histone H2B as substrates. Incubation with PP2A did dephosphorylate and reversed the activation of MBPK-2 (Fig. 5, B and D). This effect of PP2A was prevented when the incubations were performed in the presence of microcystin-LR (0.04-4 mM) or ATP (0.2 mM). Autophosphorylation and activation of MBPK-2 were unaffected by CD45 and T-cell PTPase (Fig. 5, B and D) and prior to autophosphorylation, PP2A, CD45, and T-cell PTPase had little effect, if any, on the activity of MBPK-2 (Fig. 5B). MBPK-1 and MBPK-2 exhibited little or no reaction by Western blotting with anti-phosphotyrosine antibodies before or after autophosphorylation (not shown). Beyond the level contributed by the individual enzymes, there was little or no
MBP Kinases That Phosphorylate and Activate Protamine Kinase

Effect of protein phosphatases. Purified preparations of MBPK-1 (panel A) and MBPK-2 (panel B) were incubated as shown in the absence and presence of 0.2 mM ATP and 1 mM MgCl₂ as described in the legend to Fig. 3. After 5 h at 30 °C, a 0.005-ml aliquot of the mixtures were incubated for a further 30 min in 50 mM Tris-chloride, pH 7.0, buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 14 mM β-mercaptoethanol in the absence or presence of PP2A₂ (0.1 unit), CD45 (1.5 units), or T-cell PTPase (2 units) as shown in a final volume of 0.05 ml. MBP kinase activity was then determined with a 0.005-ml aliquot of the mixtures were incubated for up to 6 h at 30 °C.

Phosphorylation and Activation of Protamine Kinase by MBPK-1 and MBPK-2. Incubation with the purified preparations of MBPK-1 and MBPK-2 resulted in the phosphorylation and activation of the protamine kinase (Fig. 7). The specific activity of the purified preparations of the protamine kinase employed in this study ranged from 4,500–18,000 units/mg. The extent of phosphorylation and activation of these preparations correlated inversely with the specific activity of the protamine kinase preparations. Thus, following incubation with MBPK-1 and MBPK-2, preparations of the protamine kinase exhibiting lower specific activities were activated to a higher degree and phosphorylated to a higher stoichiometry than preparations exhibiting higher specific activities. The range of activation was 1.5–5-fold, and the extent of phosphoryl group incorporation was 0.1–0.5 mol of phosphoryl groups incorporated per mol of the purified preparations of MBPK-1 and MBPK-2 reacted with the monoclonal antibody to MAP kinase (Fig. 6). Reaction with MBPK-1 and MBPK-2 was observed with 2 μg of this antibody. This is the recommended concentration of the antibody for the detection of the MAP kinases termed ERK-1 and ERK-2 (11, 12). At this concentration, purified preparations of the protamine kinase did not react with this antibody. Purified preparations of MBPK-1, MBPK-2, and the protamine kinase did not react with the S6 kinase antibody. Western blotting with the anti-MAP kinase antibody of freshly prepared bovine kidney extracts side by side with the purified MBPK-1 and MBPK-2 indicated that the apparent Mᵦ of these enzymes was not altered during the purification (not shown).

Catalytic Properties—Relative to MBP, purified preparations of MBPK-1 exhibited <0.1, 87, 30, 100, 5, 1, 1, 1, <0.1, and <0.1% activity with protamine sulfate, histone H1, histone H2A, histone H2B, histone H3, histone H4, casein, phosvitin, glycogen synthase a, and phosphorylase b, respectively. Relative to MBP, purified preparations of MBPK-2 exhibited <0.1, 24, 87, 100, 1, <0.1, 0.1, <0.1, and <0.1% activity, respectively, with protamine sulfate, histone H1, histone H2A, histone H2B, histone H3, histone H4, casein, phosvitin, glycogen synthase a, and phosphorylase b, respectively.

Autophosphorylation of MBPK-1 and MBPK-2 and the activities of these enzymes with MBP, histone H1, histone H2A, and histone H2B as substrates were unaffected by Ca²⁺ (0.1 mM), cGMP (0.1 mM), heparin (up to 50 μg/ml), EGTA (1 mM), Ca²⁺ (up to 0.5 mM) in the absence or presence of phosphatidylserine (40 μg/ml), or calmodulin (1 μM). Autophosphorylation of MBPK-1 and MBPK-2 and the activities of these enzymes with MBP, histone H1, histone H2A, and histone H2B as substrates were also unaffected by the synthetic peptide inhibitors of protein kinase A (TYYADFIASGRTGRRNAIHD) and protein kinase C (RFARKGALRQKNV) (each at 10 μg/ml).

Phosphorylation and Activation of Protamine Kinase by MBPK-1 and MBPK-2. Incubation with the purified preparations of MBPK-1 and MBPK-2 resulted in the phosphorylation and activation of the protamine kinase (Fig. 7). The specific activity of the purified preparations of the protamine kinase employed in this study ranged from 4,500–18,000 units/mg. The extent of phosphorylation and activation of these preparations correlated inversely with the specific activity of the protamine kinase preparations. Thus, following incubation with MBPK-1 and MBPK-2, preparations of the protamine kinase exhibiting lower specific activities were activated to a higher degree and phosphorylated to a higher stoichiometry than preparations exhibiting higher specific activities. The range of activation was 1.5–5-fold, and the extent of phosphoryl group incorporation was 0.1–0.5 mol of phosphoryl groups incorporated per mol of the purified preparations of MBPK-1 and MBPK-2 reacted with the monoclonal antibody to MAP kinase (Fig. 6). Reaction with MBPK-1 and MBPK-2 was observed with 2 μg of this antibody. This is the recommended concentration of the antibody for the detection of the MAP kinases termed ERK-1 and ERK-2 (11, 12). At this concentration, purified preparations of the protamine kinase did not react with this antibody. Purified preparations of MBPK-1, MBPK-2, and the protamine kinase did not react with the S6 kinase antibody. Western blotting with the anti-MAP kinase antibody of freshly prepared bovine kidney extracts side by side with the purified MBPK-1 and MBPK-2 indicated that the apparent Mᵦ of these enzymes was not altered during the purification (not shown).
In this paper we have described the purification to apparent homogeneity of two forms of MBP kinase designated MBPK-1 and MBPK-2 from extracts of bovine kidney cortex (Table I and Fig. 2). In addition, we showed that the purified preparations of MBPK-1 and MBPK-2 phosphorylated and activated purified preparations of the protamine kinase (Fig. 7). Together with previous observations (26), the results suggest that the mechanism by which insulin stimulated the protamine kinase in hepatocytes (26) may involve either the direct and/or indirect activation of MBPK-1 and/or MBPK-2. By analogy to other insulin-regulated protein kinases (1-6), MBPK-1 and MBPK-2 may be activated directly by phosphorylation. Indirect activation of MBPK-1 and MBPK-2 may result from the inactivation of PP2A. It is pertinent in this regard that the evidence indicates that PP2A is a specific protamine kinase-inactivating phosphatase (33). In addition, we have recently shown that phosphorylation of the catalytic subunit of PP2A on threonines by a distinct autophosphorylation-activated protein kinase inactivated this phosphatase with the protamine kinase as substrate (42, 46). However, whether this PP2A phosphorylation occurs in cells in response to insulin has not yet been determined.

The results indicate that MBPK-1 and MBPK-2 may modify the same serine(s) on the protamine kinase. This possibility is indicated by the observations that before or after incubation with PP2A the protamine kinase was phosphorylated and activated to a similar extent whether MBPK-1, MBPK-2, or a combination of these enzymes was included in the incubations. The reason the extent of the MBPK-1- and MBPK-2-mediated phosphorylation and activation of the protamine kinase correlated inversely with the specific activity of the protamine kinase preparations may be due to differences in the phosphorylation state of the protamine kinase preparations. It appears unlikely that this variation is due to different degrees of contamination with a protamine kinase present in the protamine kinase preparations because none of the protamine kinase preparations underwent autophosphorylation or activation (this study and Ref. 23). In addition, other than the protamine kinase, there was little, if any, other phosphorylation detected following incubation with MBPK-1 and MBPK-2 (Fig. 7B). In this regard, it is pertinent that, unlike MBPK-1 and MBPK-2 (Fig. 6), none of the protamine kinase preparations reacted with the MAP kinase antibody. In addition, MBPK-1, MBPK-2, and the protamine kinase did not react with antibodies that recognize the insulin- and mitogen-activated protein S6 kinases of apparent Mr = 70,000 and 90,000 (not shown). These observations are consistent with earlier studies which differentiated the protamine kinase from these as well as other protein kinases (23, 26). Because MBPK-1 and MBPK-2 partially reactivated the PP2A-inactivated protamine kinase (Fig. 7), the protamine kinase may well be regulated by multisite phosphorylation and, other than MBPK-1 and MBPK-2, distinct protamine kinase-activating kinases may exist.

The objectives of this study were to purify MAP kinase from bovine kidney cortex in order to examine the regulation of the protamine kinase. However, the properties of MBPK-1 and MBPK-2 indicate that these enzymes are different from the MAP kinases described to date. Thus, in contrast to recombinant (46-48) and immunoaffinity purified MAP kinases (49), the purified preparations of MBPK-1 and MBPK-2 underwent 50-100-fold higher autophosphorylation on threonines and no autophosphorylation on tyrosines was detected (Figs. 3 and 4). Autophosphorylation was accompanied by activation of MBPK-2 (Fig. 3B) but, by contrast to MAP kinase also (46-48), it had little or no effect on the activity of MBPK-1 (Fig. 3A). Moreover, whereas PP2Aβ, a protein serine/threonine phosphatase reversed the autophos-
phorylation of MBPK-1 and the autophosphorylation and activation of MBPK-2, the PTPases CD45 and T-cell PTPase that inactivated MAP kinase (18, 50) were without effect on MBPK-1 and MBPK-2 before or after autophosphorylation (Fig. 5). In addition, MBPK-1 and MBPK-2 did not react with anti-phosphotyrosine antibodies before or after auto-

Acknowledgments—We are grateful to Dr. Edmond H. Fischer for CD45 and T-cell PTPase and Dr. Nicholas Tonks also for a prepa-

REFERENCES